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Human cytomegalovirus control in allogeneic stem cell transplant recipients in the letermovir era – emerging humoral and cellular players

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HCMV control in allogeneic stem cell transplant recipients in the letermovir era - emerging humoral and cellular players

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Supplementary Methods

HCMV viral load measurements

HCMV DNA was quantified from EDTA plasma samples using a clinically validated real-time PCR assay, performed by the Institute of Virology at the University of Wuerzburg as part of routine diagnostics. The following primers and probe were used: CMV31 (AAgCggCCTCTgATAACCAAg), CMV32 (gAgCAgACTCTCAgAggATCgg), and CMV-TM46FAM (catgcagatctcctcaatgcggcg). A pCMV8 plasmid cloned into the pCR2.1-TOPO vector served as the positive control. PCR amplification was carried out on an ABI 7500 Real-Time PCR System (Life Technologies).

HCMV-specific IgG and IgM measurements

Serum HCMV-specific IgM and IgG levels were measured using clinically validated ARCHITECT CMV assays (Abbott, Ireland) on the ARCHITECT i2000SR system, performed by the Institute of Virology at the University of Wuerzburg. IgG samples exceeding the upper detection limit of 250 AU were automatically diluted 1:10 by the instrument and reanalyzed. In rare cases where this dilution was insufficient to bring values within range, a 1:100 dilution was prepared manually and subsequently analyzed on the same system.

Flow cytometry

After thawing, global γδ T-, B-, and NK-cell phenotypes were analyzed, and HCMV-specific B cells were quantified using flow cytometry. For γδ T- and B-cell phenotyping, 5×10^5 PBMCs per panel were stained using specific antibodies.

γδ T-cell staining consisted of TCR Vδ1 FITC, TCR γ/δ PE, CD27 PE-Vio615, TCR Vδ2 APC, TCR Vγ9 VioBlue, CD45RA VioGreen (Miltenyi, Bergisch Gladbach, Germany), CD3 AF700 (BD, Franklin Lakes, New Jersey, USA), and Fixable Viability Dye eFluor780 (Invitrogen, Waltham, Massachusetts) in 90 µL Brilliant Stain Buffer (BD). B-cell staining utilized IgG FITC, CD27 PE-Vio615 (Miltenyi), IgM BV421, IgD BV605, CD20 BV650, CD21 Alexa Fluor700 (BioLegend, San Diego, California, USA), and Fixable Viability Dye eFluor780 in 90 µL Brilliant Stain Buffer.

For the NK-cell phenotype, 5×10^5 PBMCs were washed in wash buffer (PBS + 1% FCS), blocked for 10 minutes at 4°C with 95 µL Brilliant Stain Buffer and 5 µL Blocking Reagent (Miltenyi), and stained for 30 minutes at 4°C in the same buffer. Antibodies used were CD159c PE-Vio770, CD57 APC (Miltenyi), CD56 BV510 BioLegend, CD3 AF700 (BD) and Fixable Viability Dye eFluor780.

HCMV glycoproteins were used as probes to identify specific memory B cell populations. HCMV trimer (gH/gL/gO complex, The Native Antigen Company, Kidlington, UK), pentamer (gH, gL, UL128, UL130, UL131A complex, The Native Antigen Company), and glycoprotein B (gB, SinoBiological, Beijing, China) were biotinylated with EZ-Link Sulfo-NHS-Biotin (Thermo Scientific, Waltham, Massachusetts), incubated for 30 minutes at room temperature with a 20-fold molar excess of biotin reagent, and desalted using Zeba Spin Columns (Thermo Scientific). Biotinylated proteins were enriched with 15% glycerol (final concentration) and stored at 4°C. Before use, streptavidin PE and APC (Miltenyi) were bound to the probes, and 5×10^6 PBMCs per protein/protein complex were stained. Additional antibodies used consisted of IgG FITC, CD27 PE-Vio615 (Miltenyi), IgM BV421, IgD BV605, CD20 BV650, CD21 AF700 (BioLegend), and Fixable Viability Dye eFluor780. Streptavidin BV510 (BioLegend) was used as a decoy probe to exclude non-specific B cells, with only double-positive (PE and APC) B cells considered specific. For a comprehensive, step-by-step protocol for detecting antigen-specific memory B cells, please refer to Weskamm et al[1].

Flow cytometric analysis was performed using a CytoFLEX cytometer and CytExpert v.2.4 software (Beckman Coulter, Brea, California). Data were analyzed in Kaluza v.2.1, and cell subpopulation frequencies were calculated by multiplying flow cytometry-derived percentages by absolute lymphocyte counts per µL of whole blood, as determined by clinical hematology.

References

1. Weskamm LM, Dahlke C, Addo MM. Flow cytometric protocol to characterize human memory B cells directed against SARS-CoV-2 spike protein antigens. STAR Protoc. 2022;3(4):101902.

Table S1. AlloSCT recipient characteristics: historical preemptive therapy cohort vs. current letermovir cohort

Variables	Preemptive therapy cohort (n=15)	Letermovir cohort (n=42)	*p**
Age, median (range)	55 (31-78)	62 (22-73)	0.925
Sex, n (%)			
Male	14 (93)	21 (50)	0.004**
Female	1 (7)	21 (50)	
Underlying disease, n (%)			
Chronic leukemia	0 (0)	1 (2)	
Multiple myeloma	2 (13)	2 (5)	
Acute leukemia	7 (47)	25 (60)	-
Lymphoma	0 (0)	0 (0)	
Others	6 (40)	14 (33)	
HLA matching, n (%)			
Matched related	3 (20)	5 (12)	
Matched unrelated	10 (67)	24 (57)	-
Haploidentical	0 (0)	0 (0)	
Mismatch	2 (13)	13 (31)	
Stem cell source, n (%)			
Bone marrow	0 (0)	1 (2)	>0.999
PBSC	15 (100)	41 (98)	
Conditioning Regimen, n (%)			
Reduced intensity (RIC)	14 (92)	39 (93)	>0.999
Myeloablative (MAC)	1 (8)	3 (7)	
Antithymocyte globulin, n (%)			
No	2 (13)	5 (12)	>0.999
Yes	13 (87)	37 (88)	
HCT-CI, n (%)			
0-2	8 (53)	30 (71)	
3-4	3 (20)	9 (21)	-
≥5	4 (27)	3 (7)	
aGvHD, n (%)			
0-1	11 (73)	29 (69)	>0.999
2-4	4 (27)	13 (31)	
cGvHD, n (%)			
No	9 (60)	27 (64)	0.766
Yes	6 (40)	15 (36)	
HCMV serostatus (R/D), n (%)			
+/+	14 (93)	26 (62)	0.025*
+/-	1 (7)	16 (38)	
One-year mortality, n (%)			
Alive	12 (80)	35 (83)	0.713
Dead	3 (20)	7 (17)	
HCMV reactivation, n (%)			
Controllers	11 (73)	25 (60)	0.534
csCMVi	4 (27)	17 (40)	

Figure S1.

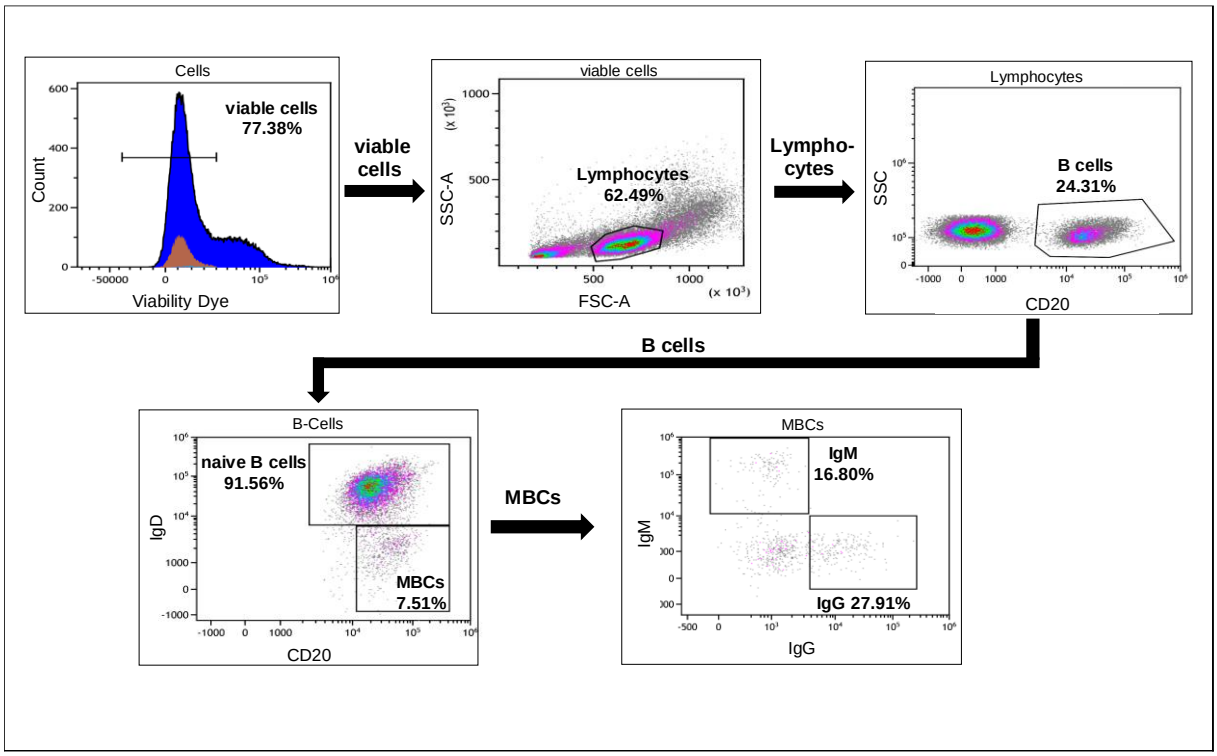
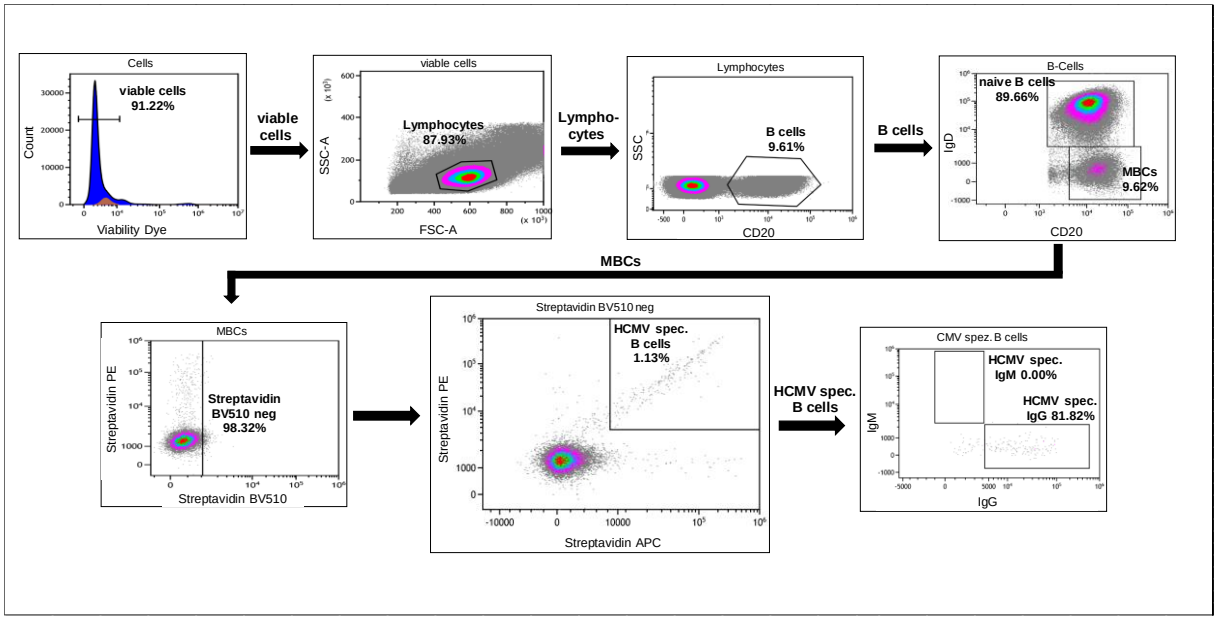


Figure S2.



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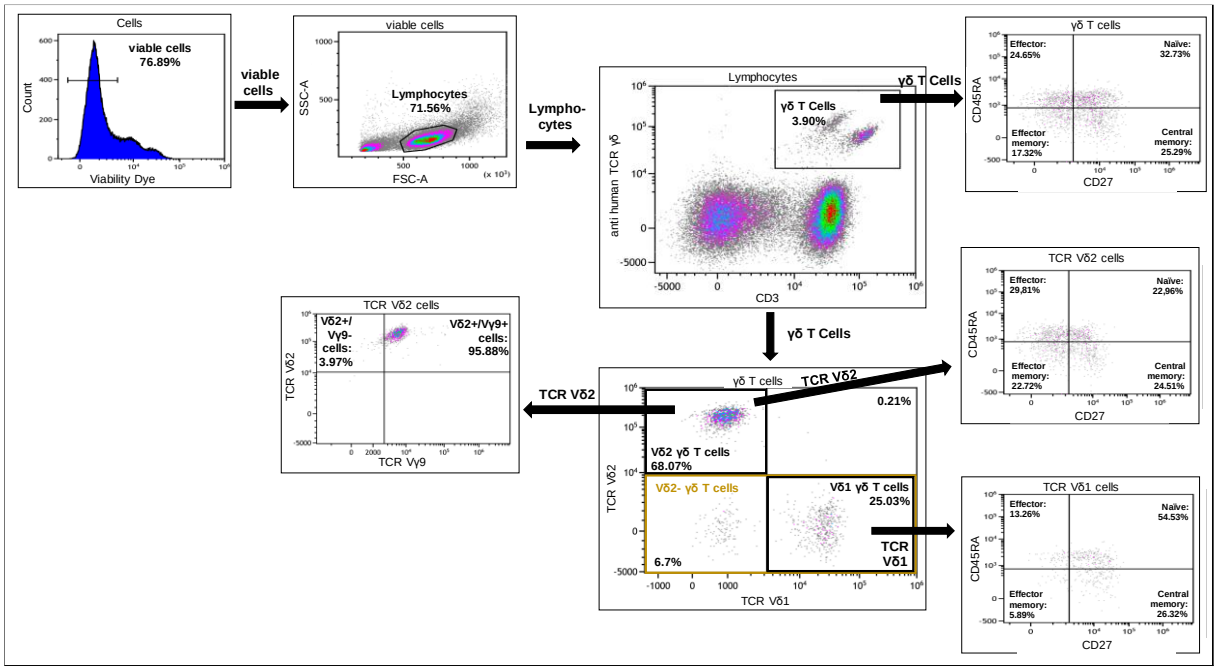

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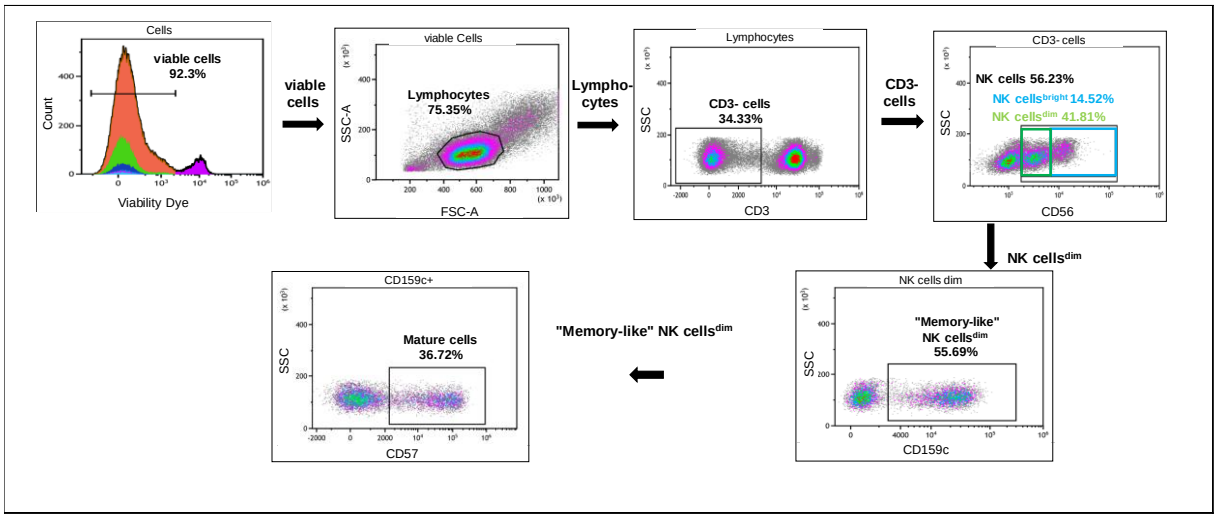


Figure S5.

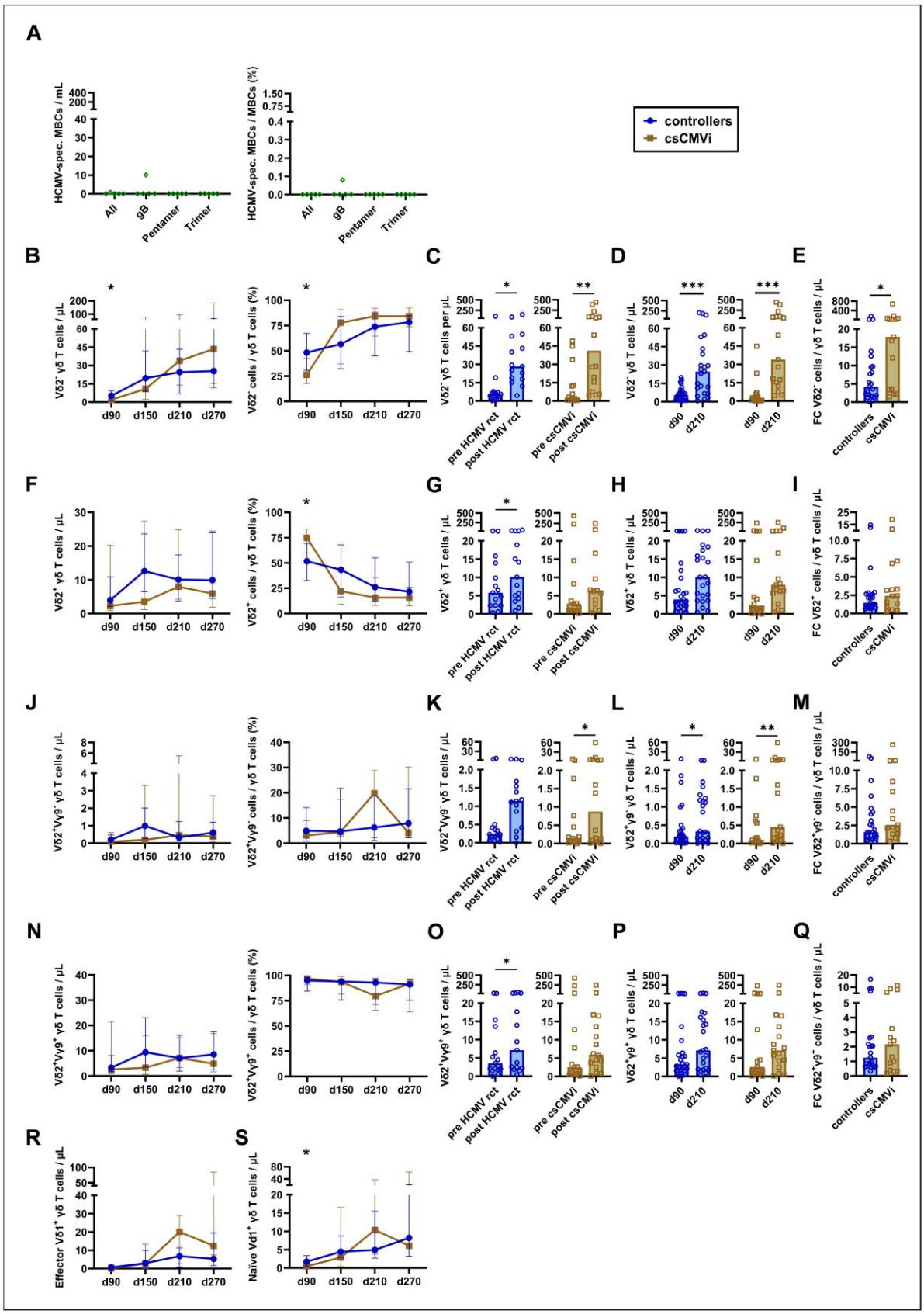

A
HCMV-spec. MBCs / mL
HCMV-spec. MBCs / MBCs (%)
All
gB
Pentamer
Trimer
controllers
csCMVi
B
Vδ2⁻ γδ T cells / µL
Vδ2⁻ cells / γδ T cells (%)
C
Vδ2⁻ γδ T cells per µL
pre HCMV rct
post HCMV rct
pre csCMVi
post csCMVi
D
Vδ2⁻ γδ T cells / µL
d90
d210
E
FC Vδ2⁻ cells / γδ T cells / µL
controllers
csCMVi
F
Vδ2⁺ γδ T cells / µL
Vδ2⁺ cells / γδ T cells (%)
G
Vδ2⁺ γδ T cells / µL
H
Vδ2⁺ γδ T cells / µL
I
FC Vδ2⁺ cells / γδ T cells / µL
J
Vδ2⁺Vγ9⁻ γδ T cells / µL
Vδ2⁺Vγ9⁻ cells / γδ T cells (%)
K
Vδ2⁺Vγ9⁻ γδ T cells / µL
L
Vδ2⁺γ9⁻ γδ T cells / µL
M
FC Vδ2⁺γ9⁻ cells / γδ T cells / µL
N
Vδ2⁺Vγ9⁺ γδ T cells / µL
Vδ2⁺Vγ9⁺ cells / γδ T cells (%)
O
Vδ2⁺Vγ9⁺ γδ T cells / µL
P
Vδ2⁺γ9⁺ γδ T cells / µL
Q
FC Vδ2⁺γ9⁺ cells / γδ T cells / µL
R
Effector Vδ1⁺ γδ T cells / µL
S
Naïve Vd1⁺ γδ T cells / µL
d90
d150
d210
d270

Figure S6.

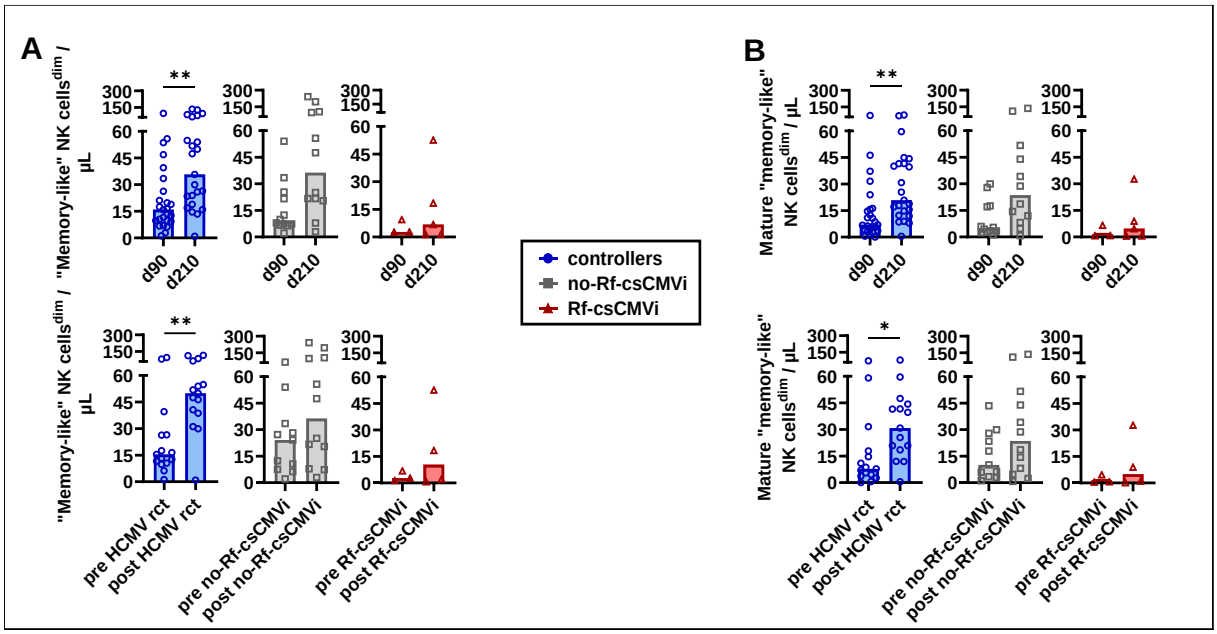
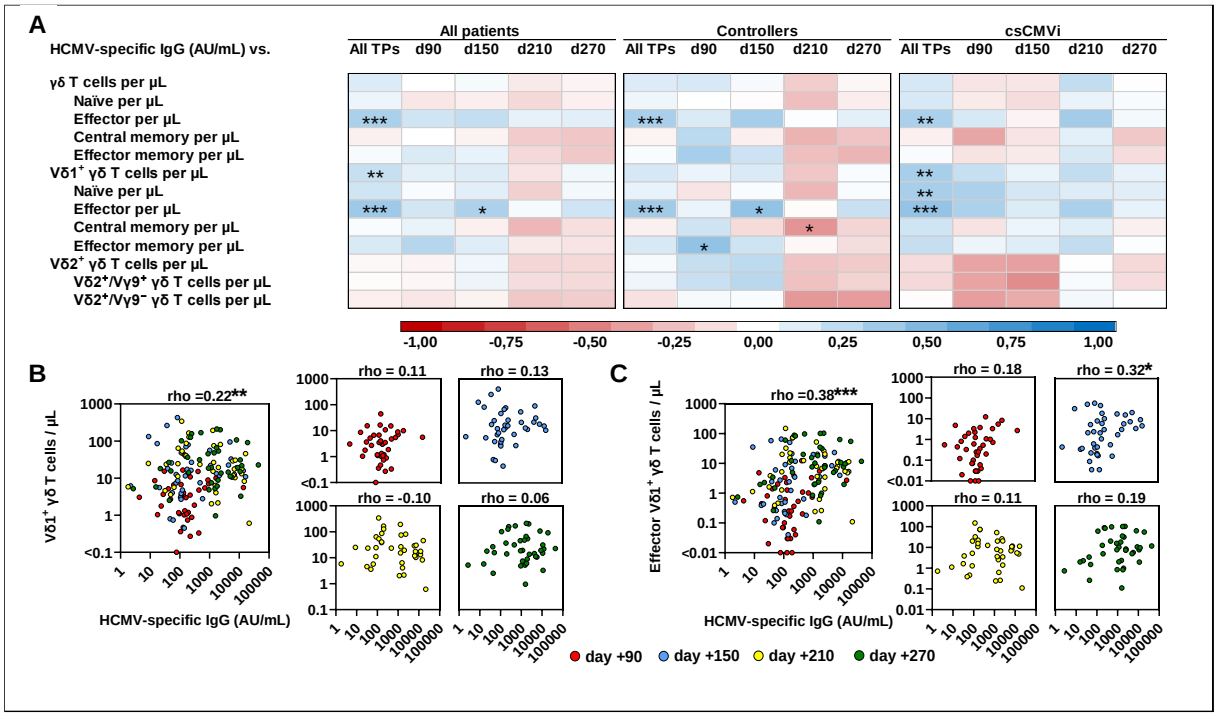

A
B
"Memory-like" NK cells^dim / µL
Mature "memory-like" NK cells^dim / µL
**
*
300
150
60
45
30
15
0
d90
d210
pre HCMV rct
post HCMV rct
pre no-Rf-csCMVi
post no-Rf-csCMVi
pre Rf-csCMVi
post Rf-csCMVi
controllers
no-Rf-csCMVi
Rf-csCMVi

Figure S7.


A
HCMV-specific IgG (AU/mL) vs.
All patients
Controllers
csCMVi
All TPs d90 d150 d210 d270
γδ T cells per µL
Naïve per µL
Effector per µL
Central memory per µL
Effector memory per µL
Vδ1+ γδ T cells per µL
Naïve per µL
Effector per µL
Central memory per µL
Effector memory per µL
Vδ2+ γδ T cells per µL
Vδ2+/Vγ9+ γδ T cells per µL
Vδ2+/Vγ9− γδ T cells per µL
-1,00 -0,75 -0,50 -0,25 0,00 0,25 0,50 0,75 1,00
B
rho =0.22**
rho = 0.11
rho = 0.13
rho = -0.10
rho = 0.06
Vδ1+ γδ T cells / µL
HCMV-specific IgG (AU/mL)
C
rho =0.38***
rho = 0.18
rho = 0.32*
rho = 0.11
rho = 0.19
Effector Vδ1+ γδ T cells / µL
HCMV-specific IgG (AU/mL)
day +90 day +150 day +210 day +270

Supplementary Table Legend

AlloSCT recipient characteristics: historical preemptive therapy cohort vs. current letermovir cohort

Mann-Whitney U test or Fisher's exact test was applied as appropriate. Abbreviations: aGvHD = acute graft versus host disease, cGvHD = chronic graft versus host disease, csCMVi = clinically significant CMV infection, HCT-CI = hematopoietic cell transplantation comorbidity index, HLA = human leukocyte antigen, PBSC = peripheral blood stem cells, R/D = recipient / donor.

Supplementary Figure Legends

Figure S1. Gating strategy for the identification of B cells.

The gating strategy used to identify B cells is depicted. Abbreviations: CD = cluster of differentiation; FSC = forward scatter; MBCs = memory B cells; IgG = immunoglobulin G; IgM = immunoglobulin M; SSC = side scatter.

Figure S2. Gating strategy for the identification of HCMV-specific memory B cells.

The gating strategy used to identify HCMV-specific memory B cells is depicted. Abbreviations: CD = cluster of differentiation; FSC = forward scatter; HCMV = human cytomegalovirus; MBCs = memory B cells; HCMV-spec. = HCMV-specific; IgG = immunoglobulin G; IgM = immunoglobulin M; SSC = side scatter.

Figure S3. Gating strategy for the identification of γδ T cells.

The gating strategy used to identify γδ T cells is depicted. Abbreviations: CD = cluster of differentiation; FSC = forward scatter; TCR = T-cell receptor; SSC = side scatter.

Figure S4. Gating strategy for the identification of ("memory-like") NK cells.

The gating strategy used to identify ("memory-like") NK cells is depicted. Abbreviations: CD = cluster of differentiation; FSC = forward scatter; TCR = T-cell receptor; SSC = side scatter.

Figure S5. HCMV-specific B cells are undetectable in HCMV-seronegative patients, and HCMV reactivation is primarily associated with alterations in the Vδ2$^-$ γδ T cell subset.

(A) Absolute numbers and frequencies of HCMV-specific memory B cells ($CD20^+IgD^-$, MBCs) targeting glycoprotein B (gB), the pentamer complex (gH/gL/UL128/UL130/UL131A), the trimer complex (gH/gL/gO), and all three antigens combined in HCMV-seronegative alloSCT recipients on day 270 (N = 5). **(B–Q)** Reconstitution of Vδ2$^-$ ($TCR\gamma\delta^+TCRV\delta2^-$) (**B-E**), Vδ2$^+$ ($TCR\gamma\delta^+TCRV\delta2^+$) (**F-I**), $V\delta2^+V\gamma9^+$ (**J-M**), and $V\delta2^+V\gamma9^-$ (**N-Q**) γδ T cells in 42 alloSCT recipients analyzed by flow cytometry. Absolute cell counts and frequencies are shown in **B**, **F**, **J**, and **N**. Mann-Whitney U test.

Cell counts before and after HCMV reactivation or clinically significant CMV infection (csCMVi) are displayed in **C**, **G**, **K**, and **O**. Paired Wilcoxon test. Changes in cell numbers between day 90 and day 210 are shown in **D**, **H**, **L**, and **P**. Paired Wilcoxon test. Intra-individual fold changes (FC) from day 90 to day 210 are presented in **E**, **I**, **M**, and **Q**. Mann-Whitney U test. (**R-S**) Absolute cell counts of effector ($CD45RA^+CD27^-$) (**R**) and naive ($CD45RA^+CD27^+$) (**S**) $V\delta1^+$ γδ T cells ($TCR\gamma\delta^+TCRV\delta1^+$) were also analyzed (paired Wilcoxon test). *p < 0.05, **p < 0.01, ***p < 0.001. Interquartile ranges are shown where applicable. Abbreviations: csCMVi = clinically significant HCMV infection; d = day; FC = fold changes; HCMV = human cytomegalovirus; rct = reactivation.

Figure S6. Failure to expand "memory-like" NK cells is linked to refractory csCMVi.

Reconstitution of NK cells in alloSCT recipients (N = 42) was analyzed by flow cytometry. (**A-B**) Evolution of "memory-like" NK $cells^{dim}$ (**A**) and mature "memory-like" NK $cells^{dim}$ (**B**) counts between days 90 and 210 (top panels), or before and after HCMV reactivation/csCMVi events (bottom panels). Paired Wilcoxon test. *p < 0.05, **p < 0.01. Abbreviations: alloSCT = allogeneic stem cell transplantation; csCMVi = clinically significant HCMV infection; d = day; rct = reactivation; Rf-csCMVi = refractory clinically significant HCMV infection.

Figure S7. HCMV-specific humoral IgG response shows a slight positive correlation to $V\delta1^+$ γδ T cells.

Correlations between HCMV-specific IgG and γδ T cells were analyzed across all timepoints (TPs) and at individual timepoints. N = 40 patients; 25 controllers, 15 csCMVi. (**A**) Heatmap summarizing correlations of HCMV-specific IgG levels with numbers of γδ T cells ($TCR\gamma\delta^+$) and their subpopulations. (**B-C**) Correlation of HCMV-specific IgG with total $V\delta1^+$ γδ T-cell ($TCR\gamma\delta^+TCRV\delta1^+$) (**B**) and effector $V\delta1^+$ γδ T-cell ($CD45RA^+/CD27^-$) numbers (**C**) in 40 patients across all timepoints (large panels) and at individual timepoints (small panels). All analyses were performed using Spearman's rank correlation test. *p < 0.05, **p < 0.01, ***p < 0.001. Abbreviations: alloSCT =

allogeneic stem cell transplantation; csCMVi = clinically significant HCMV infection; HCMV = human cytomegalovirus; Ig = immunoglobulin; TPs = timepoints.